MICROBIOLOGY 315 LABORATORY MANUAL

EXERCISE #9: EFFECTS OF CHEMICAL AGENTS AND ANTIBIOTICS ON BACTERIA; SELECTION FOR SPONTANEOUS ANTIBIOTIC-RESISTANT MUTANTS

NAME, STUDENT ID

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BIOL 315 SECTION

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INTRODUCTION

We live in a germ conscious society. We are constantly reminded of dangerous GERMS lurking in the dark corners of our homes, and in the nooks and crannies of our bodies. These GERMS threaten us with all sorts of terrible social consequences such as *BAD BREATH, DIRTY TOILETS,* and *UNDERARM ODORS!* While the above "social problems" may indeed injure your social life, they are rarely life-threatening. Nonetheless, numerous household products are touted for their efficient "germ killing" ability. In this exercise we will test some of the "germ killing" products to see just how effective they are. We will also see how doctors and hospitals determine which antibiotic to use to treat bacterial infections.

ANTISEPTICS are substances that are commonly applied to the skin, whereas **DISINFECTANTS** are substances usually used to kill microbes on inanimate objects (floor, table, sink). For example, alcohol and iodine are antiseptics often applied to the site of an injection, whereas chlorine bleach is a disinfectant for the laundry, floor, toilet, sewage, or swimming pool. **ANTIBIOTICS** are chemicals that are produced by living organisms in order to **KILL OTHER LIVING ORGANISMS.** Antibiotics are more specific and limited in the types of organisms that they kill than are antiseptics & disinfectants. For example, a given antibiotic will generally be effective in killing only some bacterial species, whereas antiseptics/disinfectants are lethal to a wide range of microbes.

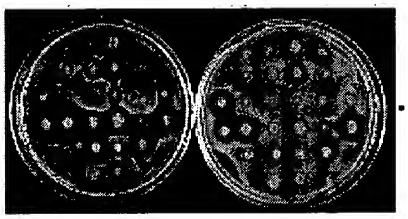


Figure 1. Different strains of bacteria were spotted onto a lawn of another bacterium. Some strains produce antibiotics that inhibit growth of this other bacterium, seen as a clear zone. Many antibiotics are discovered by

Since science demands quantification, it is necessary to assess the range and efficacy of various antimicrobial agents. One way of doing this is to place paper disks, soaked with the substance in question, on media covered with the microbe being tested in a petri dish. The petri dish is then incubated and the subsequent growth of the test species around the soaked disks is assessed. If the chemical agent being tested **INHIBITS** the test microbe there will be a clear **ZONE OF INHIBITION (ZI)** surrounding the disk where no microbial growth has occurred due to the presence of the agent. In general, the larger the diameter of the ZI, the more effective the test chemical is. Conversely, if the bacteria grow right up to the disk, the chemical is harmless. This procedure has been standardized in the case of antibiotics and other antiseptics and is the most commonly used method of assessing the efficacy of antibiotics and other antimicrobial agents against microbes. If you develop a bacterial infection the clinician

looking for microbes that produce something that inhibits other microbes.

will isolate the bacteria, spread it on an agar plate and place a series of disks soaked in various antibiotics on the plate. Following incubation, the plate will be examined for the presence of ZI around the disks. The doctor then decides which antibiotic to use to treat your infection. Newer techniques are coming into use that may allow the clinician to identify the infecting bacterium and determine its antibiotic susceptibility within a few minutes. This should allow the more timely application of correct treatment measures and save more lives. In parts 2 & 3 of this exercise, you will employ the simple but effective disk method to test several antimicrobial agents against some bacteria.

Soon after antibiotics became commonly used it was observed that if you plated several million bacteria on a medium containing a lethal dose of an antibiotic, an occasional lone colony grew. When tested, cells from such colonies were found to no longer be susceptible to that antibiotic; they were now **RESISTANT** to it. They retained this resistance even when cultivated for many generations away from the antibiotic; that is, the resistance was a genetic characteristic of that **MUTANT**. The argument raged over whether the antibiotic itself somehow "induced" mutations causing resistance or whether the resistant microbe already existed prior to exposure to the antibiotic. After a series of brilliant experiments it was proven that the latter was the case. The only role for the antibiotic was to **SELECT** for growth of the antibiotic resistant mutant cells that, **BY CHANCE** (at a very low probability of perhaps 1 in 10 million), happened to be present in the large population of cells introduced to the plate.

In part 1 of this exercise, you will select for growth of mutants resistant to an antibiotic using the **GRADIENT PLATE TECHNIQUE**. In this procedure, two tubes of melted/cooled agar media are prepared. To one of them is added the antibiotic, while nothing is added to the other. A petri dish is tilted at an

angle and the medium from the tube **WITHOUT** the antibiotic is poured into the petri dish and allowed to solidify. Then the plate is placed flat and the antibiotic-containing medium is poured on top of the first medium. After the agar solidifies, bacteria are swabbed over the surface of the plate and it is incubated. Because of the varying thickness of the antibiotic-containing medium, a gradient of antibiotic concentration is formed from near 0 to the maximum concentration. If antibiotic-resistant mutants are present, a few colonies will grow in areas of higher antibiotic concentration.

PURPOSE OF LABORATORY

1. To illustrate the inhibition of bacteria by a variety of agents.
2. To demonstrate the quantification of antibiotic efficacy.
3. To demonstrate how a physician or laboratory technician determines which antibiotic to use against an infectious bacterium.
4. To demonstrate how a spontaneous mutation can be selected for by an antibiotic.

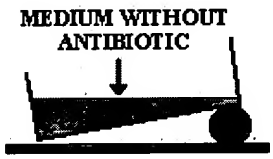
GENERAL INSTRUCTIONS

1. + Some of the materials used in this exercise are irritants or even toxic if gotten into the mouth or eyes or if left on the skin too long. Please follow the instructor's directions for handling these materials safely.
2. Obtain your bacterial strain for the antiseptic/disinfectant assays, spread it on two plates, and set them aside to dry. The explanation will follow completion of this step.
3. Following the instructor's explanation, pour the gradient plate, then set it aside to solidify as you perform the antiseptic/disinfectant assays. Just before you leave, spread the gradient plate with *E. coli* and incubate it.

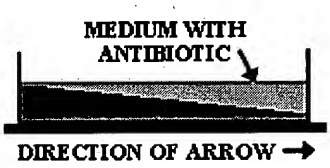
PROCEDURE

SPONTANEOUS MUTATION

1. Please examine the figures below carefully.
2. Obtain a sterile petri dish. Label it with your name. Draw an arrow in the center of the bottom of the plate (label the outside of the plate - keep it sterile inside!!).
3. Place the plate on a pencil or other object to tilt one end up with the arrow at a ***right angle*** to the object the plate is sitting on and pointing towards the object.
4. Pour a tube of the molten agar (without antibiotic) into the plate and allow it to harden. The tilt of the plate should be such that the liquid doesn't quite reach to the top edge of the angled plate.



5. When the agar has hardened (2-5 minutes), set the plate flat on the desk and add 1 ml of 1% streptomycin (the antibiotic) to the other tube of molten agar.



6. Allow plate to harden for 15 to 30 minutes. Don't jostle the plate till the agar has solidified.

7. Using a sterile swab, spread the ***E. coli*** culture evenly over the surface of the medium, being careful not to tear the agar.

8. Incubate as designated by the instructor.

9. At the next lab observe the plate for the pattern of bacterial growth. Draw a picture of it. Circle any isolated colonies growing

in a region of higher antibiotic concentration and show them to the instructor.

ANTISEPTICS/DISINFECTANTS/SELECTED MISCELLANEOUS GERM-KILLING PRODUCTS

1. Obtain two nutrient agar plates and sterile swabs. Label the plates with your name, date, section, and bacterial strain.
2. Obtain one of the bacterial cultures. Half of the class will test one strain and the other half the other.
3. Using a sterile cotton swab, spread the test bacteria evenly over the surface of the medium as demonstrated by the instructor and allow the excess liquid to soak in.
4. Allow the plates to dry at room temperature for 10 to 15 min, until the excess liquid is gone.
5. Divide the bottom of the plate into the number of sectors indicated by the instructor and label each sector as to which sample will be placed there.
6. The instructor will demonstrate how to apply the antiseptics/disinfectants etc. to the paper disks and how to lay the disks on the plate.
7. At the following lab, measure the zone-of-inhibition (ZI) diameters and draw the results in the circles below.

EVALUATION OF DISINFECTANTS (NEXT LAB)

1. Record the ZI of each of the disinfectants used in the table you prepare below.
2. Evaluate the efficacy of each disinfectant in eliminating microbes.

EFFECT OF ANTIBIOTICS

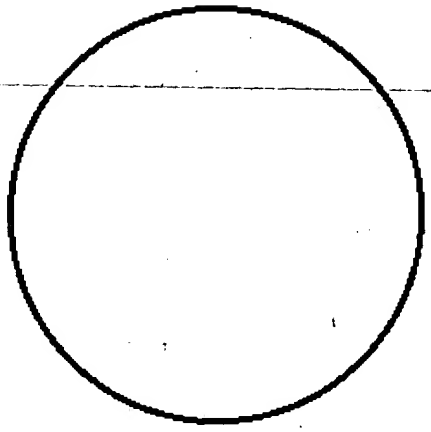
1. Read pages 93-94 in *A Photographic Atlas for the Microbiology Laboratory* before beginning this procedure.
2. Obtain the designated plate and swab it with <u>ONE</u> of the

bacterial cultures as described above.

3. Allow the plate to dry at room temperature for 10 to 15 min, until the excess liquid is gone.
4. Using forceps sterilized as demonstrated by the instructor, drop the antibiotic disk onto the agar surface as labeled. Alternatively you may use an automatic dispenser to apply the disks. The disks should be 3 cm apart and 2 cm from the edge of the plate.
5. Press the disks down with a sterile loop to "plant" them on the agar and to make effective contact.
6. Incubate plates upside down at 37°C for approximately 18 hours. The instructor will remove them and store them in the cold room until the next lab period.
7. Measure the diameter of the ZI around each disk (see this site for an example) and draw it in the circle below.
8. Examine the chart on page 94 of the ***Atlas***. Determine the susceptibility of the two bacteria to each of the antibiotics tested. Prepare a chart relating the antibiotic to ZI diameter and susceptibility of the two bacteria tested. Show this to the instructor. Compare your results with those shown in the Atlas and at this site.

DATA TABULATION

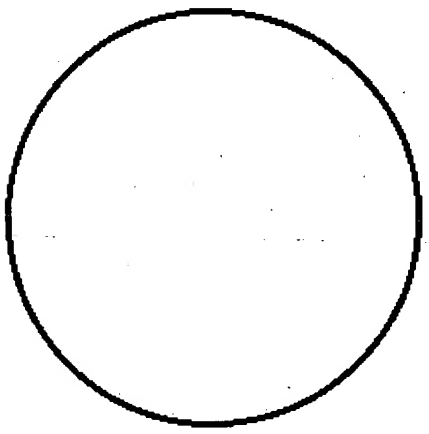
Label the plate as to orientation of the antibiotic gradient.

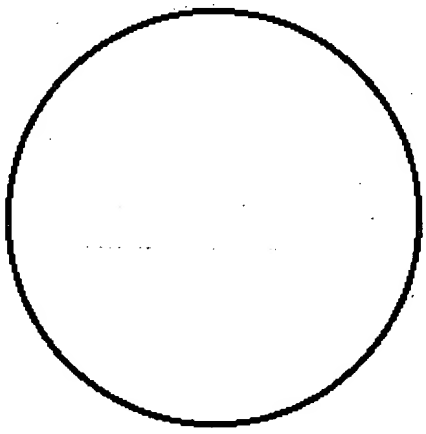


SAMPLE_____

Antiseptics and Disinfectants:

Draw a table to the right of the circles. In the first column list the agents tested; in the 2nd/3rd & 4th/5th columns label the headings with the bacteria and in the 2nd and 4th columns record the diameters of the ZI surrounding each sample next to the appropriate agent; in the 3rd and 5th columns rate the efficacy of each agent against the test bacteria using a scale of 0 = no effect to 5+ = very effective.





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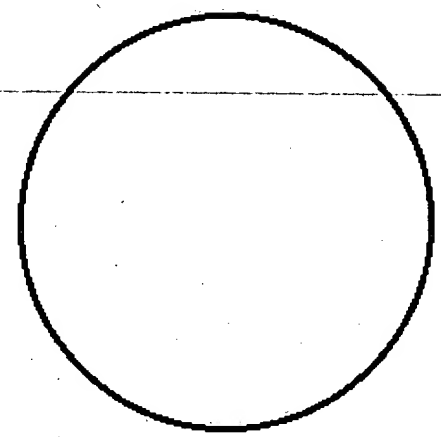
Antibiotic Susceptibility:



SAMPLE_____

SAMPLE QUESTIONS: You should be able to answer these questions at the conclusion of this laboratory.

What is the difference between an antiseptic and a disinfectant? Give an example of each.
How would you standardize or quantitate the testing of an antibiotic or other chemical as to its effect on various bacteria?
Some of the ZI on the antibiotic test plates have small colonies growing in them. What is going on here?
Can you think of a reason why a doctor might choose to use an antibiotic that produces a SMALLER ZI against a pathogen than one that produces a larger ZI?

- If you go to the doctor with the flu, what antibiotic would you expect the doctor to prescribe? Why?

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